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Induction of DT-diaphorase by 1,2-dithiole-3-thione and increase of antitumour activity of bioreductive agents

A Begleiter[1,2,3], MK Leith[1,2] and TJ Curphey[4]

[1]*Manitoba Institute of Cell Biology, Manitoba Cancer Treatment and Research Foundation; Departments of* [2]*Internal Medicine and* [3]*Pharmacology and Therapeutics, University of Manitoba, 100 Olivia Street, Winnipeg, MB, R3E 0V9 Canada;* [4]*Department of Pathology, Dartmouth College, Hanover, NH 03755, USA.*

Summary Bioreductive antitumour agents are an important new class of anticancer drugs that require activation by reduction. The two-electron reducing enzyme, DT-diaphorase, has been shown to be an important activating enzyme for the bioreductive agents, mitomycin C (MMC) and EO9. Incubation of L5178Y murine lymphoma cells *in vitro* with 1,2-dithiole-3-thione (D3T) increased the level of DT-diaphorase activity in these cells 22-fold. In contrast, D3T had no effect on the DT-diaphorase level in normal mouse bone marrow cells. Combination therapy with D3T and MMC or EO9, produced a 2- or 7-fold enhancement, respectively, of the cytotoxic activity of these antitumour agents in L5178Y cells. By comparison, D3T did not enhance the activity of MMC in marrow cells and produced only a small increase in EO9 cytotoxicity in these cells. The DT-diaphorase inhibitor, dicoumarol, inhibited the effect of D3T on the antitumour activity of the bioreductive agents, supporting the proposal that the enhanced anticancer activity was due to the elevated enzyme level. These findings suggest that D3T, or other inducers of DT-diaphorase, could be used to enhance the antitumour efficacy of bioreductive antitumour agents.

Keywords: bioreductive antitumour agent; mitomycin C; EO9; DT-diaphorase; 1,2-dithiole-3-thione

Bioreductive antitumour agents are an important new class of anticancer drugs (Workman and Stratford, 1993). They produce their antitumour activity by a variety of mechanisms, but are characterised by a requirement for reduction for activity (Adams and Stratford, 1994). As these agents are normally preferentially toxic to hypoxic cells (Workman and Stratford, 1993), there has been a major interest in using bioreductive agents in combination with radiation and drugs that kill oxygenated cells. The prototype agent in this class, mitomycin C (MMC) (Rockwell *et al.*, 1993), is widely used for the treatment of breast (Hortobagyi, 1993), gastrointestinal (Schnall and Macdonald, 1993), non-small-cell lung (Folman, 1993) and head and neck (Coia, 1993) cancers. While MMC has been used in single agent therapy, this antitumour agent generally produces higher response rates when combined with other chemotherapeutic drugs (Walters *et al.*, 1992; Coia, 1993; Folman, 1993; Hortobagyi, 1993; Schnall and Macdonald, 1993). The major toxicity associated with MMC treatment is a delayed myelotoxicity that is dose-limiting (Walters *et al.*, 1992). EO9 is an indoloquinone analogue of MMC that has shown good antitumour activity in a variety of solid tumour cells *in vitro* and *in vivo* (Hendriks *et al.*, 1993). This agent is currently in clinical trials based on its distinct antitumour profile and lack of myelosuppression (Riley and Workman, 1992).

The mechanisms involved in the activation of bioreductive antitumour agents have been extensively studied (Rockwell *et al.*, 1993; Ross *et al.*, 1993). These agents can be activated by one-electron reducing enzymes such as NADPH:cytochrome P450 reductase (EC 1.6.2.4) (Pan *et al.*, 1984; Joseph *et al.*, 1994), xanthine oxidase (EC 1.1.3.22) (Pan *et al.*, 1984) and NADH: cytochrome b_5 reductase (EC 1.6.2.2) (Hodnick and Sartorelli, 1993), and by the two-electron reducing enzymes, NAD(P)H:(quinone acceptor)oxidoreductase (EC 1.6.99.2) (DT-diaphorase) (Ross *et al.*, 1993; Plumb *et al.*, 1994) and xanthine dehydrogenase (EC 1.1.1.204) (Gustafson and Pritsos, 1992). NADPH: cytochrome P450 reductase may be the most important activating enzyme for many bioreductive agents (Keyes *et al.*, 1984; Walton and Workman, 1990), but DT-diaphorase has been shown to be an important activating enzyme for MMC (Keyes *et al.*, 1984; Begleiter *et al.*, 1989; Marshall *et al.*, 1991; Ross *et al.*, 1993), EO9 (Plumb *et al.*, 1994), the aziridinyl quinone, diaziquone (Siegel *et al.*, 1990) and other bioreductive agents (Workman and Stratford, 1993) in many systems. EO9 activity appears to be particularly sensitive to the level of DT-diaphorase under oxygenated conditions (Plumb *et al.*, 1994). In contrast, DT-diaphorase does not affect the toxicity of the benzotriazine di-*N*-oxide, tirapazamine (Patterson *et al.*, 1994), and protects cells from the toxicity of the quinone agents, menadione (Attalah *et al.*, 1988), hydrolysed benzoquinone mustard (HBM), benzoquinone mustard and benzoquinone dimustard (Begleiter and Leith, 1990).

DT-Diaphorase is a flavoprotein that catalyses two-electron reduction of quinones, quinone imines, azo dyes and other nitrogen oxides (Riley and Workman, 1992). The enzyme is ubiquitous in eukaryotes and is expressed at varying levels in most tissues (Benson *et al.*, 1980; Belinsky and Jaiswal, 1993). The enzyme is primarily located in the cytosol, but 5-10% is membrane bound in mitochondria, microsomes and Golgi apparatus (Riley and Workman, 1992). DT-diaphorase has two identical subunits with individual molecular weights of 32 kDa and requires NADH or NADPH as an electron donor for enzymatic activity (Riley and Workman, 1992). Several different DT-diaphorases have been identified in humans (Jaiswal *et al.*, 1990; Jaiswal, 1991), but the NQO_1 gene has been most extensively studied and appears to be most important for activation of bioreductive agents (Jaiswal, 1991; Riley and Workman, 1992; Belinsky and Jaiswal, 1993). The human enzymes have been shown to be very similar to other mammalian DT-diaphorases (Jaiswal, 1991). Enzyme levels may be high in liver, stomach, bladder, intestine, colon and kidney, but are usually low in haematopoietic cells (Benson *et al.*, 1980; Schlager and Powis, 1990). DT-diaphorase activity is generally higher in tumour cells compared with normal cells of the same origin and high levels of DT-diaphorase are found in some human hepatoma and colon, breast and lung carcinoma cell lines (Schlager and Powis, 1990; Belinsky and Jaiswal, 1993).

DT-diaphorase is a phase II enzyme that may play an important role in detoxifying chemically reactive metabolites,

Correspondence: A Begleiter, Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg, Manitoba, Canada R3E 0V9

thereby protecting the cell from their toxic and mutagenic effects (Beyer *et al.*, 1988; Riley and Workman, 1992). It is induced coordinately with other phase II enzymes, such as glutathione *S*-transferases, epoxide hydrolase and UDP-glucuronosyltransferases, by a variety of procarcinogens, and may be important in an early cellular defence against tumorigenesis (Beyer *et al.*, 1988; Prestera *et al.*, 1993). DT-diaphorase is also induced in many tissues by a wide variety of structurally dissimilar chemicals including 1,2-dithiole-3-thiones (D3Ts), quinones, diphenols, phenylenediamines, Michael reaction acceptors, isothiocyanates and heavy metals (Prestera *et al.*, 1993).

Recent studies have investigated using inducers of DT-diaphorase and other phase II enzymes in cancer prevention (Kelloff *et al.*, 1990). The D3T analogue, Oltipraz, which has been used for treatment of schistosomiasis (Bueding *et al.*, 1982) and is an inducer of phase II enzymes (Kensler *et al.*, 1992; Egner *et al.*, 1994), inhibits development of tumours in animals (Kelloff *et al.*, 1990; Kensler *et al.*, 1992). This agent has low toxicity in animals and is in phase I trials as a chemoprotective agent in humans (Kensler *et al.*, 1992). Other D3T analogues have also been studied for their ability to induce DT-diaphorase and other phase II enzymes (Egner *et al.*, 1994).

We have previously reported (Begleiter and Leith, 1995) that doxorubicin, a quinone-containing antitumour agent that is often used in combination regimens with MMC (Hortobagyi, 1993; Schnall and Macdonald, 1993), could selectively induce DT-diaphorase in EMT6 murine mammary tumour cells without altering the level of this enzyme in normal bone marrow cells. Combination therapy with doxorubicin and MMC produced a low level of synergistic cell kill in the tumour cells; however, this appeared to be unrelated to the induction of DT-diaphorase.

In the current study we investigated whether D3T could selectively induce DT-diaphorase in L5178Y murine lymphoma cells compared with normal marrow cells from DBA/2 mice, and examined whether combining this agent with MMC or EO9 in an appropriate schedule could produce enhanced antitumour activity *in vitro*.

Materials and methods

Materials

Fischer's media and horse serum were obtained from Gibco BRL (Grand Island, NY). Methocult M3430 and M5300 and all related marrow culture supplies were from Stem Cell Technologies (Vancouver, Canada). All reagents for the DT-diaphorase assay were from Sigma (St Louis, MO), as was MMC. EO9 was kindly supplied by Dr HR Hendriks, New Drug Development Office, European Organization for Research and Treatment of Cancer, Amsterdam, The Netherlands. Mesh filters (100 μm) were from VWR (Mississauga, Canada). Protein concentration was measured using the Bio-Rad DC Kit (Bio-Rad, Mississauga, Canada) with gamma globulin as standard. MMC was dissolved in phosphate-buffered saline (PBS), while EO9 was dissolved in dimethyl sulphoxide (DMSO):ethanol (1:1, v/v). The final concentration of DMSO or ethanol did not exceed 1%. Fischer's media with 12% horse serum and supplemented with 10 mM Hepes to maintain pH was used for all incubations with MMC or EO9.

Cells

L5178Y mouse lymphoma cells were grown in suspension in Fischer's medium with 12% horse serum. All experiments were performed with logarithmically growing cells. Normal mouse marrow cells were isolated from the femurs of 10–12 week old DBA/2 mice. Mice were asphyxiated with CO_2 and both femurs were removed. Marrow was obtained by flushing the femurs with media using a 22 gauge needle and marrow from the femurs of 2–3 DBA/2 mice was pooled. A single cell suspension was ensured by passing the cells through a 25 gauge needle and any particulate matter remaining was removed by passing through a 100 μm mesh nylon filter. The cells were spun at 450 *g* for 10 min and resuspended in Methocult M5300.

Induction of DT-diaphorase activity

L5178Y or marrow cells were incubated at 37°C for up to 48 h with 75 μM D3T. At various times, aliquots of cells were washed twice with PBS, resuspended in 200 μl of 0.25 M sucrose, sonicated three times for 10 s on ice and stored at −80°C.

Protein concentration was determined in all samples with the Bio-Rad DC Protein Assay kit using bovine gamma globulin as standard. For L5178Y cells, 20–100 μg protein was assayed for DT-diaphorase activity, while for marrow cells 40–80 μg was assayed. DT-diaphorase activity was measured in the sucrose sonicates by a modification of the procedure of Prochaska and Santamaria (1988) using a Cary 1 spectrophotometer (Varian). DT-diaphorase activity was expressed as nmol MTT reduced min^{-1} mg $protein^{-1}$.

Cytotoxicity assay for L5178Y cells

L5178Y cells were incubated at 37°C for 40 h with 0 or 75 μM D3T. Media was removed and the cells were washed and incubated with various concentrations of MMC or EO9 for 1 h. For studies involving inhibition of DT-diaphorase by dicoumarol, 100 μM dicoumarol was added to the media 15 min prior to addition of the antitumour agent. Drug cytotoxicity was determined by clonogenic assay as described previously (Begleiter *et al.*, 1989). Cloning efficiencies averaged 36.9±2.6%. The surviving cell fraction was determined and the D_{10} (concentration of drug required to reduce the surviving cell fraction by 90%) was obtained from the linear regression line of the drug concentration vs surviving cell fraction curve. The surviving cell fractions were the mean of 5–9 experiments. The D_{10} values for different treatments were compared by a *t*-test comparing the significance of the differences of the slopes of the linear regression lines. The concentrations of D3T and dicoumarol used in these studies were not toxic to the cells.

Cytotoxicity assay for DBA/2 mouse marrow cells

Marrow cells were obtained as described above. Cells were incubated with 0 or 75 μM D3T at 37°C for 40 h. Cells were washed, resuspended in media and incubated with various concentrations of MMC or EO9 at 37°C for 1 h. Cells were washed and resuspended in 1 ml of Methocult M5300. For control cultures, 1.5×10^4 to 3×10^4 cells were added to 1 ml of Methocult M3430 following the protocol provided by Stem Cell Technologies. For drug-treated cells, increased cell numbers up to 6×10^4 were added to 1 ml of Methocult M3430. Total colonies (CFU-G, CFU-M and CFU-GM) on the plates were counted after 7–10 days and the surviving cell fraction and the D_{10} was determined as described for L5178Y cells. The surviving cell fractions were the mean of three or four experiments. The D_{10} values for different treatments were compared by a *t*-test comparing the significance of the differences of the slopes of the linear regression lines. The concentration of D3T used in these studies was not toxic to the cells.

Results

Induction of DT-diaphorase by 1,2-dithiole-3-thione

Initial DT-diaphorase levels were low in both L5178Y and normal marrow cells. When L5178Y lymphoma cells were treated *in vitro* with 75 μM D3T DT-diaphorase activity increased linearly for approximately 24 h and reached a peak

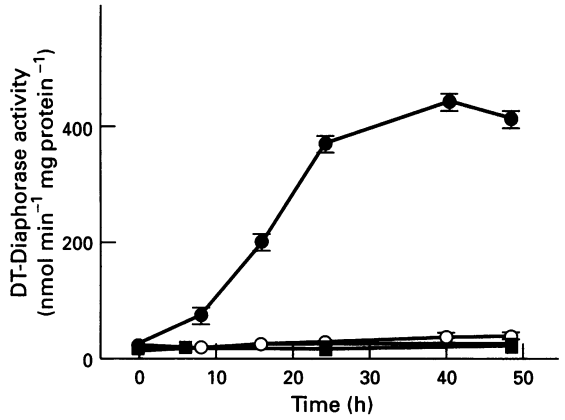

Figure 1 Induction of DT-diaphorase by D3T in L5178Y and normal murine marrow cells. L5178Y (○, ●) or normal marrow (□, ■) cells were incubated at 37°C with (●, ■), or without (○, □), 75 μM D3T and at various times the level of DT-diaphorase activity was measured. The points represent the mean ± s.e.m. of three experiments.

level at 40 h (Figure 1). The level of enzyme activity in the tumour cells increased from 20 nmol min^{-1} mg $protein^{-1}$ to 440 nmol min^{-1} mg $protein^{-1}$. In contrast, D3T had no effect on DT-diaphorase activity in normal marrow cells (Figure 1). In addition, D3T did not alter NADPH:cytochrome P450 reductase activity in the L5178Y cells.

Combination therapy with D3T and MMC

L5178Y cells were incubated *in vitro* with, or without, 75 μM D3T for 40 h and then were treated with MMC for 1 h. Pretreatment with D3T decreased the D_{10} for MMC in these cells from 1.31 μM to 0.67 μM ($P<0.001$) (Table I, Figure 2a). Addition of dicoumarol prior to MMC treatment significantly inhibited D3T enhancement of antitumour activity ($P<0.002$) (Table I, Figure 2a). In contrast, D3T did not significantly increase the cytotoxic activity of MMC in normal marrow cells (Figure 2b).

Table I Combination therapy with D3T and MMC or EO9 in L5178Y cells

	D_{10} (μM)[a]		
Drug	*Drug alone*	*Drug + D3T*	*Drug + D3T + dicoumarol*
MMC	1.31 ± 0.04	0.67 ± 0.04*	1.09 ± 0.09**
EO9	5.28 ± 0.22	0.79 ± 0.06*	3.23 ± 0.49**

[a] Mean ± s.e.m. *$P<0.001$ *vs* drug alone. **$P<0.002$ *vs* drug + D3T.

Combination therapy with D3T and EO9

L5178Y cells were incubated *in vitro* with, or without, 75 μM D3T for 40 h and then were treated with EO9 for 1 h. L5178Y cells treated with D3T were 7-fold more sensitive to EO9 than cells treated with EO9 alone ($P<0.001$) (Table I, Figure 3a). Addition of dicoumarol prior to treatment with EO9 significantly inhibited D3T enhancement of EO9 antitumour activity ($P<0.001$) (Table I, Figure 3a). Pretreatment of normal marrow cells with D3T produced a small increase in the sensitivity of these cells to EO9 (Figure 3b).

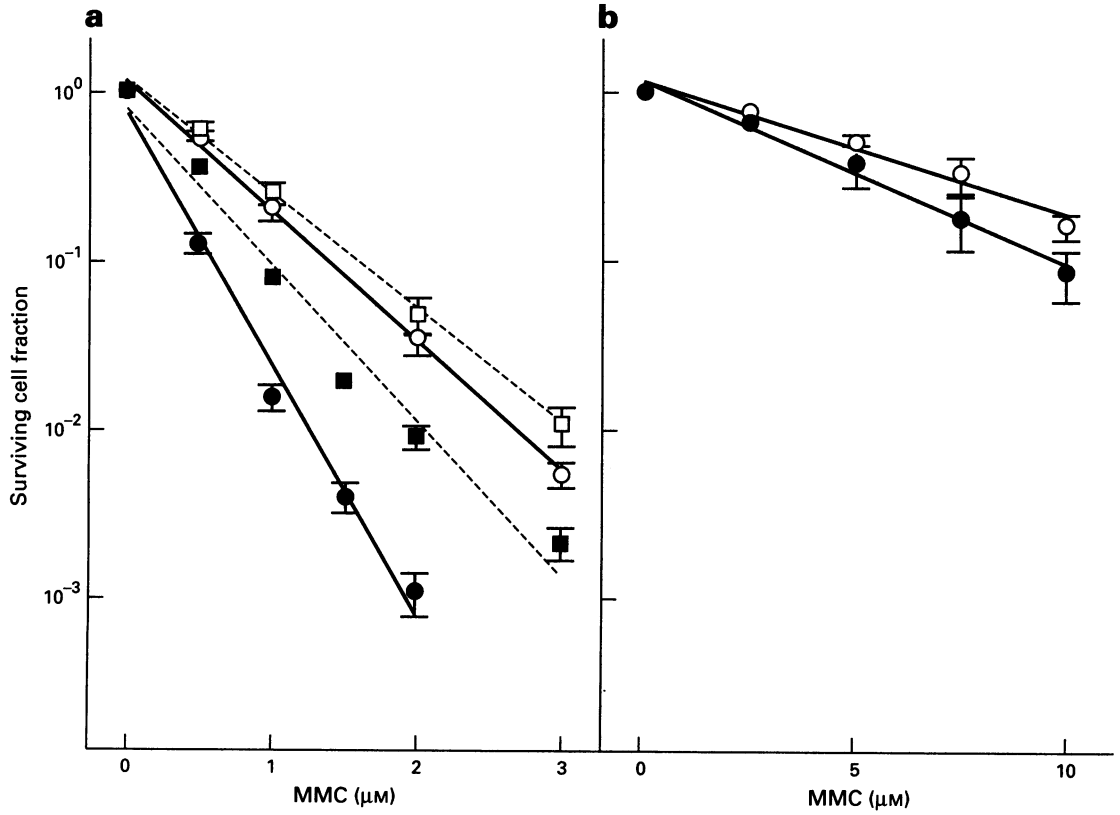

Figure 2 Combination therapy with D3T and MMC in L5178Y (**a**) and normal murine marrow cells (**b**). Cells were incubated at 37°C with (●, ■), or without (○, □), 75 μM D3T for 40 h. Cells were then treated with various concentrations of MMC for 1 h (○, ●), or with 100 μM dicoumarol for 15 min and then with various concentrations of MMC for 1 h (□, ■). The points represent the mean ± s.e.m. of 3–7 experiments. The lines are linear regression lines.

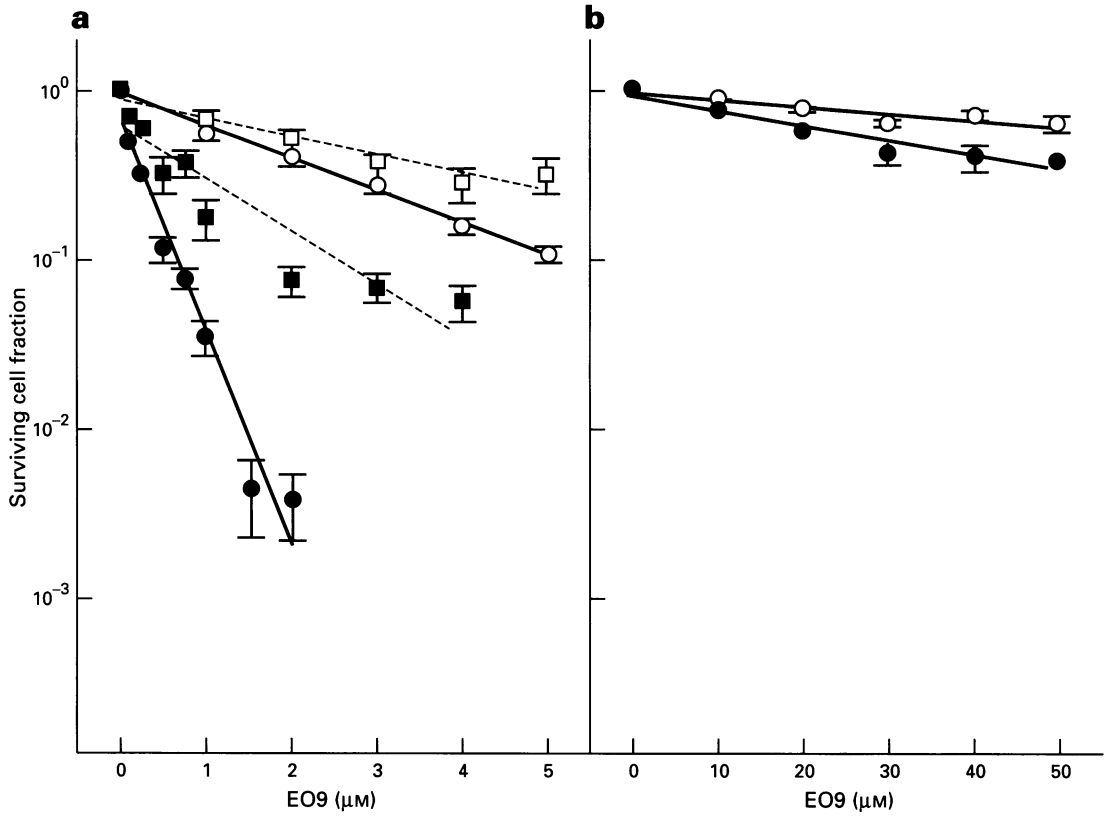

Figure 3 Combination therapy with D3T and EO9 in L5178Y (**a**) and normal murine marrow cells (**b**). Cells were incubated at 37°C with (●, ■), or without (○, □), 75 μM D3T for 40 h. Cells were then treated with various concentrations of EO9 for 1 h (○, ●), or with 100 μM dicoumarol for 15 min and then with various concentrations of EO9 for 1 h (□, ■). The points represent the mean ± s.e.m. of 4–9 experiments. The lines are linear regression lines.

Discussion

In this study we demonstrated that D3T could selectively induce DT-diaphorase in tumour cells compared with normal marrow cells. *In vitro* treatment with a non-toxic concentration of D3T increased the level of DT-diaphorase in L5178Y murine lymphoma cells 22-fold without effecting the enzyme level in normal murine marrow cells. Previous studies have shown that D3T induced a 4-fold increase in DT-diaphorase activity in murine hepatoma cells (Egner *et al.*, 1994) and a 10-fold increase in enzyme activity in human myeloid leukaemia cells (Li *et al.*, 1994). Twerdok *et al.* (1992) demonstrated a 2- to 3-fold increase in DT-diaphorase activity in primary bone marrow stromal cells from DBA/2 and C57Bl/6 mice treated *in vitro* with 75 μM of the 5-(2-pyrazinyl) analogue of D3T; however, *in vivo* feeding of DBA/2 mice with 0.1% of this D3T analogue in their diet produced only a 30% and 50% increase in DT-diaphorase in whole bone marrow and primary stromal cells respectively (Twerdok *et al.*, 1993).

It is not clear why we observed selectivity in the induction of DT-diaphorase in the tumour cells compared with the normal marrow cells, but this may relate to an intrinsic difference between the tumour and normal cells. It has been suggested that DT-diaphorase inducers may induce transcription of the enzyme by producing a redox signal that, through Ref-1, or a related nuclear protein, may modify the DNA binding domain of the early response genes, *Jun* and *Fos*. Increased affinity of the *Fos–Jun* complex for an AP1 site may then produce greater transcription of DT-diaphorase (Belinsky and Jaiswal, 1993; Yao *et al.*, 1994). Thus, differences between tumour and normal cells in one or more of the elements in this pathway may account for the selective induction observed in this study.

Elevated levels of DT-diaphorase activity have been shown to increase the cytotoxic activity of MMC (Keyes *et al.*, 1984; Begleiter *et al.*, 1989; Ross *et al.*, 1993) and EO9 (Riley and Workman, 1992; Plumb *et al.*, 1994). Thus, we investigated whether pretreatment of L5178Y cells with D3T to increase the level of DT-diaphorase could potentiate the antitumour activity of these bioreductive agents. Combination therapy with D3T resulted in a 2-and 7-fold enhancement of MMC and EO9 cytotoxicity, respectively, in the tumour cells. In contrast, D3T treatment produced only a small increase in the toxicity of these agents in normal murine marrow cells. The enhanced cytotoxicity by MMC in L5178Y cells pretreated with D3T is similar to the increased activity of MMC in L5178Y/HBM10 cells (Begleiter *et al.*, 1989). The L5178Y/HBM10 cell line, which was developed from L5178Y cells by exposure to the model quinone agent, HBM, has a 24-fold increased level of DT-diaphorase compared with the parent cell line (Begleiter *et al.*, 1988).

The results of this study are consistent with our hypothesis that the antitumour efficacy of bioreductive agents that are activated by DT-diaphorase can be enhanced by selectively increasing the level of this enzyme in tumour cells. The role of DT-diaphorase in the increased antitumour activity of MMC and EO9 was confirmed by the finding that the DT-diaphorase inhibitor, dicoumarol, significantly reduced the effect of D3T on the cytotoxicity of these agents. Dicoumarol also inhibited the activity of the bioreductive agents in the absence of D3T, but this effect was smaller than that observed in the presence of D3T and likely represents the effect of inhibiting activation of MMC and EO9 by the base level of DT-diaphorase in the L5178Y cells. We were unable to fully reverse the effect of D3T in the L5178Y cells with dicoumarol. This may reflect an inability to completely inhibit DT-diaphorase activity with the concentration of dicoumarol used. Higher levels of dicoumarol could not be used in these studies as they were toxic to the cells. The use of dicoumarol as an inhibitor of DT-diaphorase activity should be viewed with some caution as dicoumarol can also inhibit other activating enzymes, such as NADH-

cytochrome b_5 reductase (Hodnick and Sartorelli, 1993) and it has produced conflicting effects in some studies *in vitro* (Rockwell *et al.*, 1989).

We previously reported that DT-diaphorase could be induced in EMT6 murine mammary tumour cells by the antitumour agent, doxorubicin, without induction of this enzyme in normal bone marrow cells from Balb/c mice (Begleiter and Leith, 1995). However, the increased level of DT-diaphorase in the EMT6 cells did not appear to enhance the antitumour activity of MMC. There are several possible explanations for this discrepancy. The base level of DT-diaphorase in the EMT6 cells was high and the level of enzyme activity was increased only 40% after induction. Thus, the extent of induction may have been too low to produce a significant effect on MMC cytotoxicity, or the base level of DT-diaphorase in the cells may have been so high that an increase in enzyme activity did not result in any additional drug activation. Alternatively, the variation in the two studies may be due to differences in the types of tissues used. Additional studies with other tumour types, having different base levels of DT-diaphorase, are required to distinguish between these possibilities.

In summary, this study has demonstrated that D3T can selectively induce DT-diaphorase in murine L5178Y lymphoma cells compared with normal murine bone marrow cells. Combination therapy with D3T and the bioreductive antitumour agents, MMC and EO9, produced significantly increased tumour cell kill that was due to the elevated levels of DT-diaphorase. Similar treatment of normal marrow cells with D3T and bioreductive agent produced only a small increase in cytotoxicity in these cells suggesting that D3T, or other inducers of DT-diaphorase, might be used to enhance the antitumour efficacy of bioreductive antitumour agents.

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